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Assessment of cytotoxic effects and disruption of biological functions induced by heavy metals in the black cutworm, *Agrotis ipsilon*

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Abstract

Background Heavy metal pollution poses risks to terrestrial food chains. We evaluated biochemical and cellular responses of fifth-instar *Agrotis ipsilon* (black cutworm) fed castor (*Ricinus communis*) leaves collected from two polluted sites and a reference site.

Methods Oxidative damage markers (protein carbonyls and lipid peroxides), antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), and glutathione S-transferase (GST)], the defense-related enzyme phenoloxidase (PO), and metallothionein (MT) were measured in the midgut and brain tissues. Midgut ultrastructure was examined by transmission electron microscopy (TEM). Heavy metals [cadmium (Cd), chromium (Cr), nickel (Ni), copper (Cu), lead (Pb), and zinc (Zn)] were quantified in plant and larval tissues.

Results Larvae fed polluted leaves showed marked oxidative damage and altered enzyme activities. Notable bioaccumulation included midgut Cd increases of ~ 1540% (Group 1) and ~ 1740% (Group 2) versus control; brain MT and GST were also elevated (GST in brain ≈ 3× control). TEM revealed mitochondrial lysis, microvillar disorganization and chromatin condensation in midgut cells. Correlation analysis linked specific metals (e.g., Cu, Zn, Cd) to antioxidant responses.

Conclusions Dietary heavy metals cause pronounced biochemical and ultrastructural injury in *A. ipsilon* larvae, supporting their potential use as terrestrial bioindicators.

Keywords Heavy metals, Oxidative stress, Antioxidant enzymes, Metallothioneins, Histopathology, *Agrotis ipsilon*

Introduction

Anthropogenic activities significantly impact natural systems, complicating the assessment of stress effects on species and ecosystems due to varying toxin levels and differential organismal sensitivities to foreign substances (Ray, 2016). Metals from industrial processes and vehicle emissions harm ecosystems, with contaminants like metal-laden insecticides and fertilizer-induced heavy metals contributing to soil degradation (Hamzeh et al., 2011; Tariq et al., 2016; Zhao et al., 2018). These pollutants not only affect soil quality but also compromise food safety. Organismal susceptibility to heavy metal (HM)

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toxicity is influenced by exposure duration, developmental stage, and genetic factors (Tchounwou et al., 2012). While essential at physiological levels, excessive HMs can trigger the production of reactive oxygen species (ROS), resulting in oxidative stress (Israr et al., 2011). Insects, in particular, are vulnerable to cadmium exposure from contaminated plants, experiencing physiological damage that can result in mortality (Shu et al., 2015). Cadmium disrupts cellular processes and interferes with antioxidant defenses, while lead exposure induces oxidative stress, ionic imbalance, and potential cell death (Vlahogianni et al., 2007; Wang et al., 2021). Zinc, though essential, can be toxic at elevated levels, especially for herbivores (Jin et al., 2020).

Long-term or mild stress from environmental pollutants triggers the production of protective proteins in animals, mitigating damage and activating defense mechanisms (Augustyniak et al., 2009). Insects, integral to food chains, accumulate toxins, making their response to pollutants crucial for understanding environmental impacts. Arthropods handle metals by sequestering them in lipid vesicles or binding them to proteins like metallothioneins (Apostolopoulou et al., 2023). In polluted areas, insects capable of managing metal exposure may survive (Valko et al., 2016). Robust antioxidant systems are key to tolerance in metal-rich environments, protecting organisms from metal-induced oxidative damage (Ercal et al., 2001). Insects use small molecules and enzymes in their defense against chemical damage (Blagojević & Grubor-Lajšić, 2000; Kodrík et al., 2015).

Water and soil insects directly encounter HMs in their habitats, while herbivorous insects ingest HMs through contaminated plants. These metals penetrate plant roots via active (symplastic) or passive (apoplastic) transport mechanisms (Rashid et al., 2023), and their phytotoxicity depends on transport and accumulation within the plant (Yan et al., 2020; Soliman et al., 2024a, b). Plants utilize metal transporters for absorption, translocation, and detoxification (Islam et al., 2020), and metal interactions, such as mercury's antagonism against arsenic absorption, affect accumulation in plant tissues (Kafle et al., 2022). Soil insects in polluted areas employ detoxification strategies, such as activating metallothioneins (Hauser-Davis et al., 2020; Choi et al., 2008), but less is known about herbivorous insect responses to HM pollution. These insects typically store HMs in their midgut, which serves as both a barrier and a habitat (Ballan-Dufrançais, 2002; Pigino et al., 2005).

Heavy metals can significantly impact various physiological aspects of insects, including growth, developmental processes, reproductive success, and egg viability (Cervera et al., 2004; Sildanchandra & Crane, 2000; Wu et al., 2006). Exposure to these metals can impair metabolic functions, increase oxygen consumption (Ortel &

Vogel, 1989), and trigger oxidative stress (Zaman et al., 1994; Cervera et al., 2003). It has been suggested that heavy metals can cross the blood–brain barrier in vertebrates (Gilgun-Sherki et al., 2001), thereby inducing oxidative stress and leading to disruptions in redox signaling within the brain (Cobley et al., 2018). Insect models, such as *Drosophila melanogaster*, are susceptible to metal toxicity, exhibiting neurological, behavioral, and cellular responses (Peterson et al., 2017; Nas & Çolak, 2020; Fasae & Abolaji, 2022). These species-specific effects emphasize the need for reliable insect markers to assess heavy metal pollution in terrestrial and aquatic environments. Studies on insect mineral storage structures, including lysosomes and spherocrystals, have highlighted their potential as general indicators of heavy metal exposure, regardless of species (Pawert et al., 1996; Ballan-Dufrançais, 2002). However, research on the ultrastructure of metal storage organs, such as the midgut and Malpighian tubules, remains limited.

The black cutworm (*Agrotis ipsilon*) is a widely distributed, polyphagous noctuid pest that readily ingests leaves from diverse crop and wild plant species; its broad diet and ecological prevalence make it a realistic consumer in agroecosystems potentially exposed to contaminated irrigation and industrial run-off (Jin et al., 2023; Rasmy et al., 2024). Despite extensive work on metal effects in model dipterans and aquatic invertebrates, less is known about tissue-specific bioaccumulation and linked biochemical and ultrastructural responses in herbivorous Lepidoptera under field-relevant exposure (Pallottini et al., 2023). Here we address this gap by testing the hypothesis that dietary exposure to heavy metals (from field-collected castor (*Ricinus communis*) leaves) leads to (i) tissue-specific bioaccumulation, (ii) elevated oxidative damage and altered antioxidant responses, and (iii) ultrastructural midgut pathology consistent with metal-induced cytotoxicity. Our explicit objectives were to quantify plant and larval metal loads, measure oxidative and detoxification biomarkers in midgut and brain tissues, and relate biochemical changes to ultrastructural midgut damage.

Materials and methods

Sample collection

Castor leaf samples for larval feeding were collected from three sites. Two polluted sites were selected: Site A (29.7648721° N, 31.3114177° E), influenced by emissions from local brick kilns, and Site B (29.7482685° N, 31.3162675° E), impacted by effluents from the Helwan Company for Fertilizers and Chemical Industries, Cairo, Egypt. A reference site (30.0264630° N, 31.2057960° E), located in the Cairo University gardens, served as the control, irrigated exclusively with tap water (Rasmy et al., 2024). Wastewater from the polluted sites is often released into nearby watercourses, which are then used

to irrigate agricultural lands growing crops such as cabbage, sesame, okra, beans, clover, and corn.

Insect

The *A. ipsilon* colony was maintained at the insectary of the Entomology Department, Faculty of Science, Cairo University. Newly hatched larvae were reared on *R. communis* leaves harvested from two industrially affected locations (Sites A and B) and an uncontaminated reference site. The leaves were carefully washed with ultrapure water before being used in the experiment to remove any potential surface contaminants. Third instar individuals were individually housed in plastic containers (20×20×30 cm) to prevent cannibalistic behaviour and maintained under controlled insectary conditions (27±1 °C, 10 h light : 14 h dark photoperiod, and 60±10% relative humidity). Adult moths were kept under the same environmental conditions and provided with a 10% sugar solution as a food source in glass jars.

Metal analysis in castor leaves and insects

Heavy metal analysis (Cd, Cr, Ni, Cu, Pb, and Zn) in castor leaves and *A. ipsilon* larval tissues (midgut, brain, and whole body) was performed following the methodology established by Soliman et al. (2024a), with results documented by Rasmy et al. (2024). Table 1 provides a concise overview of the degree of heavy metal contamination in castor bean leaves and insect body and tissues of contaminated sites relative to the control site.

Sample preparation for biochemical analyses in the herbivore *A. ipsilon*

A more detailed description of the experimental procedures for estimating oxidative stress markers and enzyme activities, including specific reagent concentrations and additional methodological steps, is provided in the Supplementary Information. Briefly, brain and gut tissues were carefully dissected from the larvae. These tissues were then homogenized in an ice-cold phosphate buffer (pH 7.0), formulated with specific additives to stabilize the samples and preserve enzymatic activity during the extraction process. For clarity, all biochemical assays reported here were performed on $n=3$ biological replicates per site, where each biological replicate comprised pooled tissue from 30 larvae. Homogenates were used for analysis, and protein content for all samples was determined with the Bradford method (1976). A brief overview of the procedure and analyses is provided below.

Biomarkers of oxidative stress

Protein carbonyls

The quantification of protein carbonyls was conducted following the procedure outlined by Levine et al. (1990).

Protein carbonyls in the samples react with 2,4-dinitrophenylhydrazine (DNPH) to form a stable adduct. Subsequently, this was analyzed with spectrophotometry at 366 nm, and the protein carbonyl levels were reported as optical density per milligram of protein.

Lipid peroxides

Lipid peroxidation was quantified (μmol/mg protein) by determining thiobarbituric acid-reactive substances (TBARS) following the procedure outlined by Ohkawa et al. (1979). The absorbance at 532 nm was recorded to determine the malondialdehyde levels, a key indicator of lipid peroxidation.

Antioxidant enzyme assays

Superoxide dismutase (SOD)

The activity of SOD was measured following the protocol of Misra and Fridovich (1972). The method involves monitoring the reduction of epinephrine at 480 nm, where changes in absorbance correspond to SOD activity. The enzyme's activity was expressed as the change in optical density per minute per milligram of protein.

Catalase (CAT) activity

To measure CAT activity, the method outlined by Aebi (1984) was employed. The decrease in absorbance at 240 nm, associated with the decomposition of hydrogen peroxide, was monitored. CAT activity was determined by calculating the change in optical density (OD) per minute per milligram of protein.

Ascorbate peroxidase (APOX)

The activity of APOX was determined following Asada (1984). The reaction was initiated by adding hydrogen peroxide (H₂O₂), and the change in absorbance at 290 nm was measured over 30 s. Enzyme activity was quantified as the change in optical density per minute per milligram of protein.

Glutathione S-transferase (GST)

GST activity was determined using the method by Habig et al. (1974), which monitors the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. The rate of conjugation was quantified and expressed as enzyme activity in micromoles per milligram of protein per minute (μmol/mg protein/min), as described by Suganya et al. (2016).

Phenol oxidase (PO) activity

The activity of PO was assessed by the method outlined by Gomes et al. (2003). This method involves tracking the oxidation of L-DOPA to dopachrome at 490 nm. Enzyme activity was calculated in units (U), where one

unit corresponds to the quantity of enzyme needed to catalyze the oxidation of 1 μmol of L-DOPA per minute, following the protocol by Genta et al. (2010).

Metallothionein (MT) assay

Metallothionein levels in the brain and midgut tissues of *A. ipsilon* larvae were measured using extraction and assay protocols based on those described by Klein et al. (1990) and Sun et al. (2007). Larvae in the fifth instar were dissected, and the tissues were carefully washed with phosphate-buffered saline (pH 8.0), then dried and weighed (30 larvae per replicate, with three replicates). The tissues were homogenized in 10 mM Tris–HCl buffer (pH 8.6) on ice, followed by centrifugation at $12,000\times g$ for 10 min at 4 °C. The supernatant was then boiled for 4 min, after which it underwent a second centrifugation at $10,000\times g$ for 20 min at 4 °C. The supernatant was combined with ice-cold ethanol in a 3:1 ratio and left to precipitate overnight at –20 °C. Following precipitation, the sample was centrifuged again at $10,000\times g$ for 20 min at 4 °C, and the pellet was resuspended in 5 mL of 10 mM Tris–HCl buffer (pH 8.6). After incubating for 3 h, the suspension was centrifuged once more at $15,000\times g$ for 20 min at 4 °C. The final supernatant (100 μL) was mixed with 600 μL of 10 mM Tris–HCl buffer (pH 8.6), 100 μL of 20 mM CdCl_2 , and 100 μL of 2% hemoglobin solution. The mixture was incubated at room temperature for 10 min, followed by heating to boiling for 2–3 min. After centrifugation at $12,000\times g$ for 10 min at 4 °C to eliminate heat-sensitive proteins, the cadmium concentration in the supernatant was quantified using atomic absorption spectrophotometry (AAS) with a Hitachi instrument (Japan), in accordance with the method outlined by Shu et al. (2012).

Light and transmission electron microscopy of *A. ipsilon* midgut cells

The larval midgut was chosen for both light and transmission electron microscopy (TEM) examinations, as it exhibited relatively higher metal concentrations than other tissues. The midgut was fixed in a 2.5% glutaraldehyde solution in phosphate buffer (pH 7.2) at 4 °C for 3 h. Following fixation, the samples were rinsed in the same phosphate buffer and post-fixed in a 1% osmium tetroxide solution in phosphate buffer (pH 7.2) at 4 °C for 1 h. Dehydration was performed using a graded series of acetone solutions (50%, 70%, 80%, and 90%) at room temperature, with each concentration applied for 15 min. The final dehydration step utilized 100% acetone at 4 °C for 20 min. After dehydration, the specimens were embedded in Spurr resin and allowed to dry under a vacuum. For light microscopy, semi-thin sections were prepared and stained with toluidine blue. For TEM, ultrathin sections were obtained using an ultramicrotome (Nova

ultralome, LKAB, Sweden), placed on copper grids, and stained with uranyl acetate and lead citrate. The specimens were then analyzed and photographed with a transmission electron microscope (JEOL JEM 1400) operating at 80 kV.

Data analysis

Before statistical analyses, data transformations were performed to meet underlying assumptions. Protein carbonyl content, CAT activity, PO activity, and MT levels were subjected to Box-Cox transformation. The data were initially assessed for homogeneity of variance using both Levene's and F tests, while normality was evaluated with the Shapiro-Wilk test. A two-way ANOVA was conducted to examine the effects of site (reflecting varying pollution levels) and tissue type (midgut vs. brain) on oxidative stress markers, including protein carbonyls, lipid peroxides, and metallothionein (MT) levels. To explore the combined effect of site and tissue type on antioxidant enzyme activity (including SOD, CAT, APOX, GST, and PO), a multivariate analysis of variance (MANOVA) was performed. Bonferroni corrections were applied to adjust for multiple comparisons in the two-way and multivariate ANOVAs, reducing the risk of Type I errors. Pearson's correlation coefficient was used to investigate the relationships between various physiological parameters in the 5th instar *A. ipsilon*, including heavy metal concentrations, oxidative stress biomarkers, enzyme activities (SOD, CAT, APOX, GST, PO), and MT levels. A correlation heatmap was generated using the Seaborn library in Python. All statistical analyses were carried out using IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA), with a significance threshold set at $p < 0.05$.

Results

Heavy metal levels in plant and larval tissues

Analysis of heavy metal concentrations in castor leaves (*R. communis*) and *A. ipsilon* larval tissues revealed significant accumulation of contaminants in both plant material and insect tissues from experimental sites compared to the reference site (Table 1).

Castor leaves from the experimental sites showed elevated levels of all six heavy metals studied. Group 2 sites exhibited considerably higher contamination than Group 1 sites for most metals. The most dramatic increases were observed in Cr concentrations, with an extraordinary 1780% increase in Group 2 and a 276% increase in Group 1 compared to the reference site. Ni also showed substantial accumulation, with 684% and 466% increases in Groups 2 and 1, respectively. Cd (346%, 138%), Pb (119%, 95%), Zn (64%, 40%), and Cu (269%, 398%) also showed significant elevations, with Cu uniquely displaying higher concentrations in Group 1 than in Group 2.

Heavy metal accumulation in *A. ipsilon* larvae fed contaminated leaves revealed tissue-specific bioaccumulation patterns. The most dramatic whole-body accumulation was observed for Ni in Group 2 (1013% increase), followed by Pb (3993% increase). Group 1 larvae showed comparatively modest increases in whole-body metal content, with the highest increase being Pb (151%). The midgut exhibited exceptional accumulation of Cd in both Group 1 (1540% increase) and Group 2 (1740% increase). Zn also accumulated substantially in gut tissue, with 570% and 754% increases in Groups 1 and 2, respectively. Cu showed a notable difference between Group 1 (43% increase) and Group 2 (1040% increase). The most striking finding was the extraordinary accumulation of Cd in Group 1 brain tissue (4483% increase), which was significantly higher than in Group 2 (850% increase). Zn also showed remarkable brain accumulation in Group 2 (965% increase), while Cr demonstrated substantial increases in both groups (550% and 1050% for Groups 1 and 2, respectively).

Analysis of tissue-specific metal distribution revealed distinct accumulation patterns. The midgut appeared to be the primary site of accumulation for Cd and Zn, particularly in Group 2 larvae. Brain tissue showed preferential accumulation of Cd (especially in Group 1), Cr, and Zn. These tissue-specific accumulation patterns are summarized in Table 1.

Biomarkers of oxidative damage

Protein carbonyls

The effects of site (pollution levels) and tissue type (midgut vs. brain) on protein carbonyls in *A. ipsilon* were analyzed using two-way ANOVA. Significant main effects were observed for both site and tissue type, but their interaction was insignificant. The site of leaf collection had a highly significant impact on protein carbonyl levels ($F(2,11)=87.344$, $p<0.001$, $\eta^2 = 0.941$). Larvae consuming leaves from polluted sites (Site A and Site B) displayed elevated protein carbonyl levels compared to those fed leaves from the control site. Protein carbonyl

concentrations increased progressively from the control site to Site A and Site B, indicating that higher pollution levels exacerbate protein oxidation. Tissue type also significantly influenced protein carbonyl levels ($F(1,11)=164.164$, $p<0.001$, $\eta^2 = 0.937$). Protein carbonyl levels were consistently higher in midgut compared to the brain across all sites, reflecting a higher oxidative burden in the digestive tissue (Fig. 1). The interaction between site and tissue type was not statistically significant ($F(2,11)=0.893$, $p=0.437$, $\eta^2 = 0.140$). This suggests that the impact of pollution levels on protein carbonyl levels is comparable between the midgut and brain tissues, without any pronounced differential effects.

Lipid peroxides

The two-way ANOVA evaluated the effects of site (pollution levels) and tissue type (midgut vs. brain) on lipid peroxidation in *(A) ipsilon*. Significant main effects were observed for both factors and significant interaction between them. The site of leaf collection had a highly significant impact on lipid peroxidation ($F(2,11)=89.421$, $p<0.001$, $\eta^2 = 0.937$). Larvae fed leaves from polluted sites exhibited markedly higher lipid peroxidation levels than those fed leaves from the control site. Mean values for lipid peroxidation increased progressively from the control site to Site A and then Site (B) Tissue type significantly influenced lipid peroxidation ($F(1,11)=220.615$, $p<0.001$, $\eta^2 = 0.948$). Lipid peroxidation levels were consistently higher in the midgut compared to the brain, across all sites. A significant interaction between site and tissue was identified ($F(2,11)=7.640$, $p=0.007$, $\eta^2 = 0.560$). Post hoc comparisons revealed that lipid peroxidation in the midgut was significantly higher in larvae fed leaves from Site B compared to Site A and the control site. In the brain, lipid peroxidation also increased with pollution levels, but the differences between Site A and the control were not statistically significant ($p=0.121$) (Fig. 1).

Table 1 Percentage change in heavy metal concentrations in castor leaves and *Agrotis ipsilon* tissues (midgut, brain, whole body) at group 1 and group 2 relative to the reference site (normalized to 100%)

Metal	Plant		Insect					
			Whole body		Gut		Brain	
	Gp1	Gp2	Gp1	Gp2	Gp1	Gp2	Gp1	Gp2
Zn	+40%	+64%	+25%	+91%	+570%	+754%	+256%	+965%
Pb	+95%	+119%	+151%	+3993%	+487%	+487%	+157%	+500%
Ni	+466%	+684%	+40%	+1013%	+93%	+417%	+118%	+277%
Cr	+276%	+1780%	+125%	+416%	+187%	+562%	+550%	+1050%
Cd	+138%	+346%	+15%	+377%	+1540%	+1740%	+4483%	+850%
Cu	+398%	+269%	+33%	+35%	+43%	+1040%	+340%	+460%

Data derived from pooled samples ($n=3$ biological replicates; each replicate = 30 larvae)

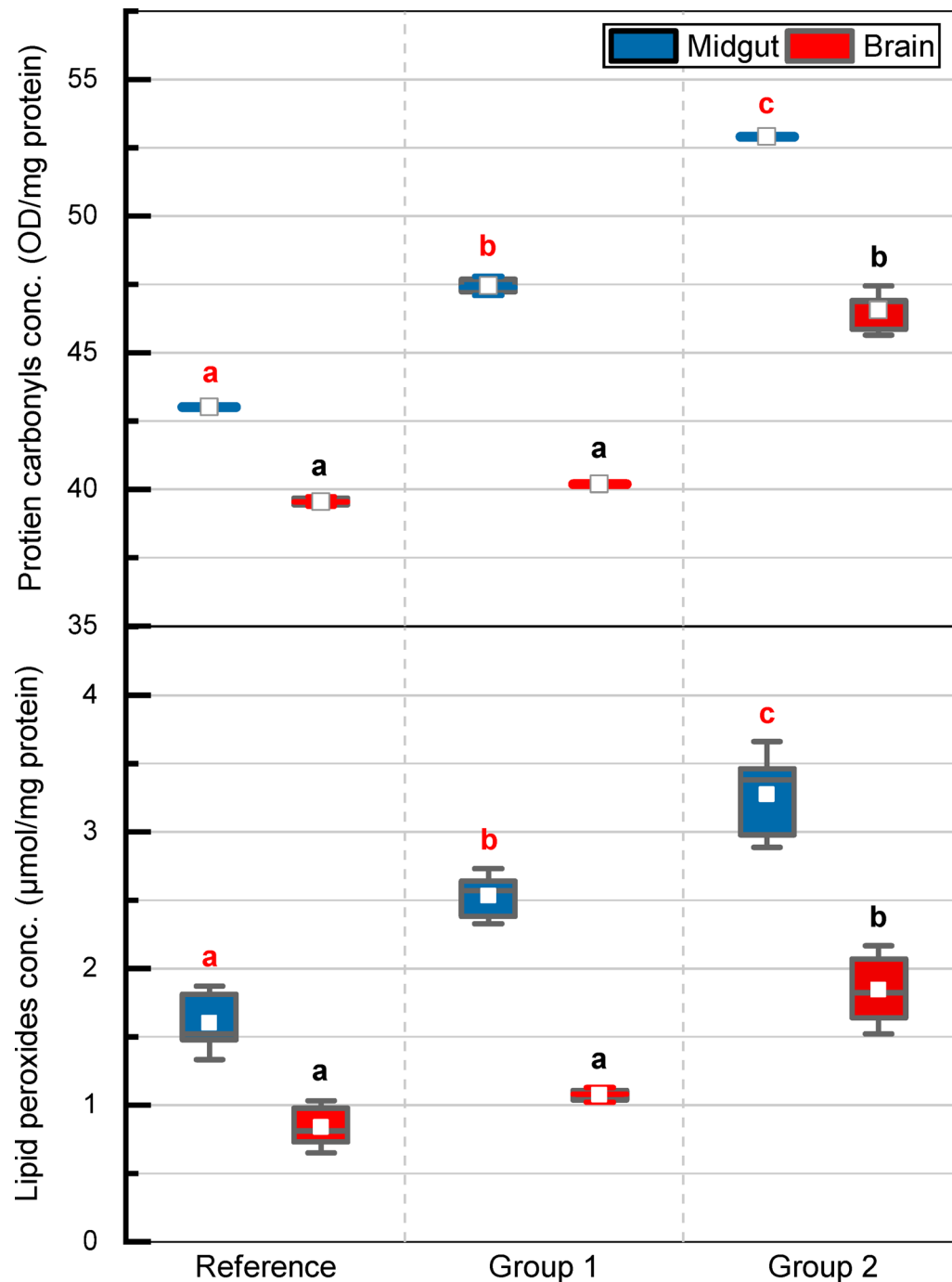


Fig. 1 Protein carbonyls (OD/mg protein) and lipid peroxides (μmol/mg protein) in midgut and brain homogenates from 5th-instar *Agrotis ipsilon* larvae fed leaves from the reference site (Control), Site A (Group 1) and Site B (Group 2). Data are mean ± SE ($n=3$ biological replicates; each replicate=pooled tissue from 30 larvae). Statistical analysis: Two-way ANOVA (effects of site and tissue); protein carbonyls — $F(2,11)=87.344$, $p<0.001$; tissue effect $F(1,11)=164.164$, $p<0.001$. Lipid peroxides — $F(2,11)=89.421$, $p<0.001$; interaction $F(2,11)=7.640$, $p=0.007$. Post-hoc: Bonferroni test; different letters indicate significant differences ($p<0.05$)

Antioxidant enzymes and phenoloxidase

The multivariate analysis of variance (MANOVA) revealed significant main effects of both location (Wilks' $\lambda=0.000$, $F(10,14)=108.172$, $p<0.001$, partial $\eta^2=0.987$) and tissue type (Wilks' $\lambda=0.004$, $F(5,7)=327.367$,

$p<0.001$, partial $\eta^2=0.996$) on enzyme activities. Additionally, a significant interaction between location and tissue type was observed (Wilks' $\lambda=0.003$, $F(10,14)=22.465$, $p<0.001$, partial $\eta^2=0.941$).

Subsequent univariate analyses showed significant effects for both main factors and their interaction on most enzymes. Superoxide dismutase (SOD) activity showed significant differences across locations ($F(2,11)=106.949$, $p<0.001$, partial $\eta^2 = 0.951$) and tissues ($F(1,11)=182.035$, $p<0.001$, partial $\eta^2 = 0.943$), with a significant location $\times$ tissue interaction ($F(2,11)=17.134$, $p<0.001$, partial $\eta^2 = 0.757$). SOD levels were consistently higher in the midgut compared to the brain, with the highest activities observed at Site B (polluted site) for both

tissues (Fig. 2). Catalase (CAT) activity demonstrated significant variation among locations ($F(2,11)=40.418$, $p<0.001$, partial $\eta^2 = 0.880$), but unlike other enzymes, showed no significant difference between tissues ($F(1,11)=2.550$, $p=0.139$) and no significant interaction effect ($F(2,11)=1.741$, $p=0.220$). CAT activity decreased progressively from control to Site B, suggesting a possible inhibition under increased pollution stress. Ascorbate peroxidase (APOX) showed significant effects of location ($F(2,11)=135.239$, $p<0.001$, partial $\eta^2 = 0.961$) and tissue

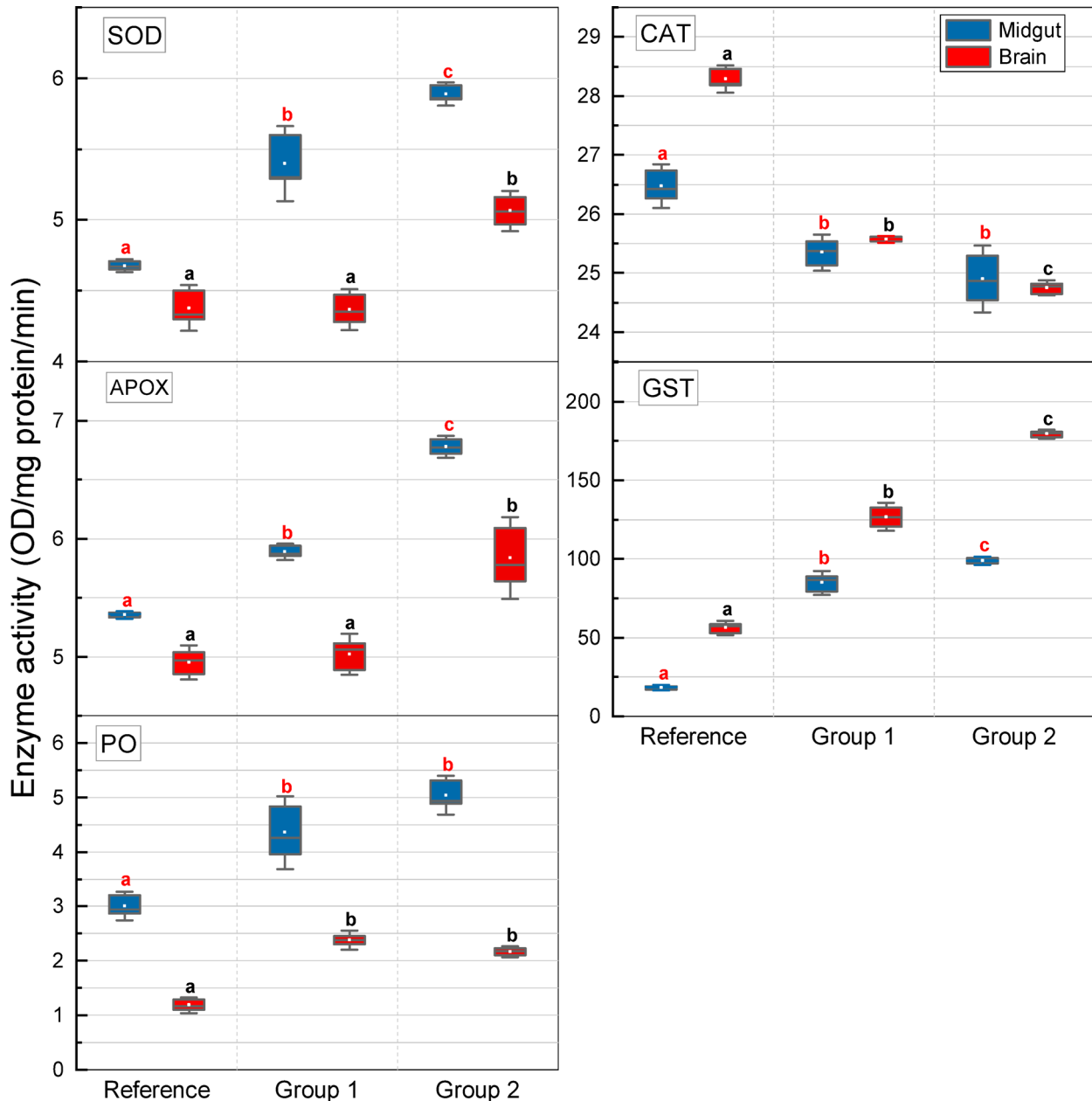


Fig. 2 Antioxidant enzyme activities (SOD, CAT, APOX, GST, PO) measured in midgut and brain tissues of 5th-instar *Agrotis ipsilon* after 5 days of feeding on leaves from Control, Group 1 and Group 2. Data are mean $\pm$ SE ($n=3$ biological replicates; each replicate = pooled tissue from 30 larvae). Multivariate analysis (MANOVA) tested combined enzyme responses across sites and tissues; Bonferroni corrected pairwise tests are shown on the panels ($p < 0.05$). Units and assay methods are in Methods

type ($F(1,11)=152.517$, $p<0.001$, partial $\eta^2 = 0.933$), with a significant interaction ($F(2,11)=7.209$, $p=0.010$, partial $\eta^2 = 0.567$). APOX activity was consistently higher in midgut tissue and showed the highest elevation at Site B, particularly in midgut samples. Glutathione S-transferase (GST) demonstrated the most pronounced differences among all enzymes studied, with highly significant effects of location ($F(2,11)=1073.995$, $p<0.001$, partial $\eta^2 = 0.995$) and tissue type ($F(1,11)=875.001$, $p<0.001$, partial $\eta^2 = 0.988$), and a significant interaction ($F(2,11)=60.834$, $p<0.001$, partial $\eta^2 = 0.917$). Brain tissue consistently showed higher GST activity than midgut tissue, with dramatic increases observed at both polluted sites. The most striking elevation was observed in brain tissue from Site B, where GST activity was approximately three times higher than control levels. Phenoloxidase (PO) activity showed significant effects of location ($F(2,11)=334.325$, $p<0.001$, partial $\eta^2 = 0.984$) and tissue type ($F(1,11)=1195.032$, $p<0.001$, partial $\eta^2 = 0.991$), with a significant interaction ($F(2,11)=105.039$, $p<0.001$, partial $\eta^2 = 0.950$). PO activity was consistently higher in midgut tissue compared to brain tissue, with significant elevations observed at both polluted sites (Fig. 2).

Metallothionein (MT) content

Analysis of metallothionein (MT) content revealed significant main effects of both sampling location ($F(2,11)=45.386$, $p<0.001$, partial $\eta^2 = 0.892$) and tissue type ($F(1,11)=10.261$, $p=0.008$, partial $\eta^2 = 0.483$). The interaction between location and tissue type was insignificant ($F(2,11)=1.491$, $p=0.267$, partial $\eta^2 = 0.213$). MT levels were consistently elevated in larvae that consumed leaves from polluted sites compared to those from the control site. Specifically, in midgut tissue, MT content increased from 237 mg/kg at the control site to 298.33 mg/kg at Site A and 311.67 mg/kg at Site B. Similarly, in brain tissue, MT levels rose from 248 mg/kg at the control site to 302.33 mg/kg at Site A and 327.33 mg/kg at Site B. MT content was consistently higher in brain tissue compared to gut tissue at each site (Fig. 3).

Correlation analysis of heavy metals, oxidative stress, and antioxidant enzymes

The correlation analysis revealed complex interrelationships among antioxidant enzymes, oxidative stress markers, metallothionein levels, and heavy metal concentrations in the fifth instar *A. ipsilon* (Fig. 4). Notably

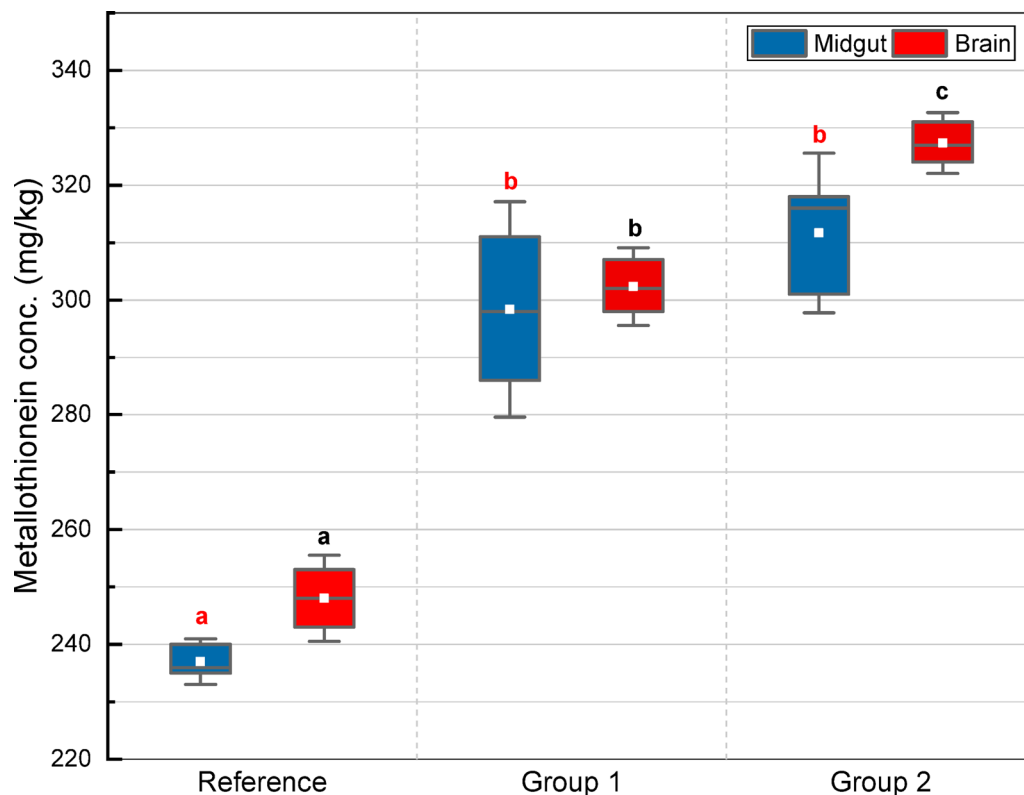


Fig. 3 Metallothionein (MT) concentrations in midgut and brain tissues of fifth-instar *Agrotis ipsilon* larvae after 5 days of feeding on castor leaves collected from the reference site (Control), Site A (Group 1) and Site B (Group 2). MT is expressed as mg kg⁻¹ tissue dry weight. Data are shown as mean $\pm$ SE ($n=3$ biological replicates; each replicate=pooled tissue from 30 larvae). Two-way ANOVA: site effect $F(2,11)=45.386$, $p<0.001$; tissue effect $F(1,11)=10.261$, $p=0.008$. Post-hoc comparisons (Bonferroni test, $p<0.05$): different red letters above midgut boxes denote statistically significant differences among groups; different black letters above brain boxes denote statistically significant differences among groups

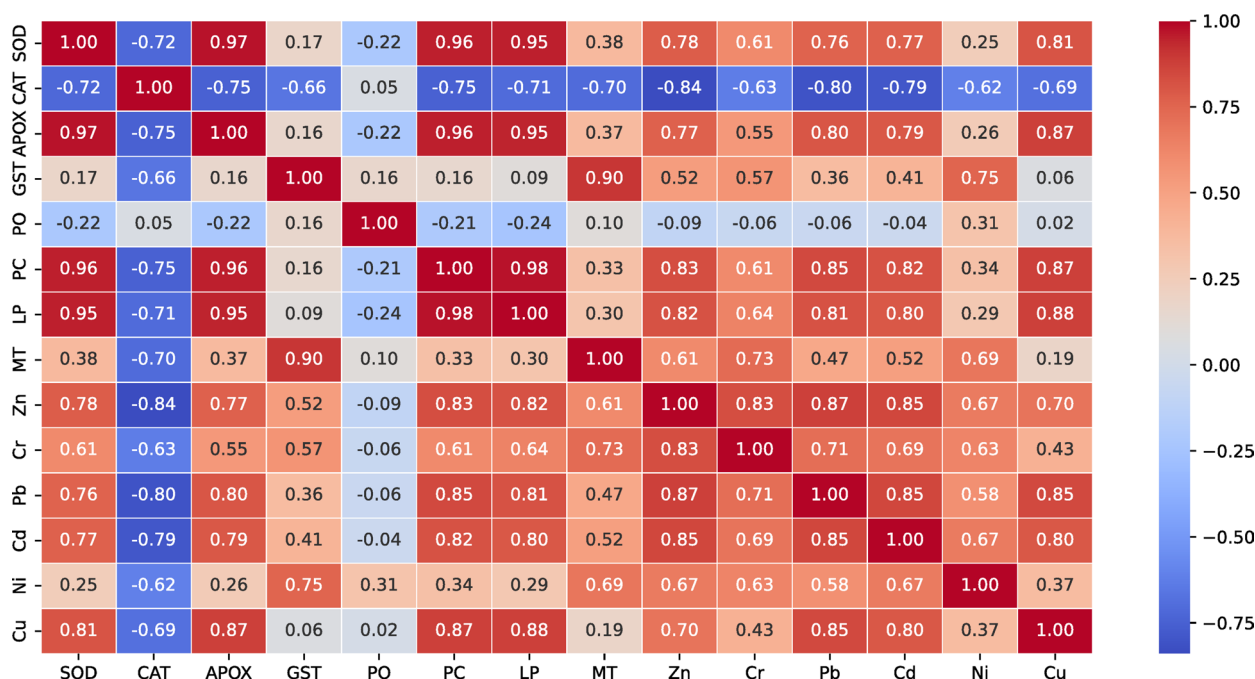


Fig. 4 Correlation heatmap illustrating the relationships between heavy metal concentrations (Zn, Cu, Cr, Ni, Cd, and Pb), oxidative stress markers (protein carbonyl [PC] and lipid peroxides [LP]), antioxidant enzyme activities (superoxide dismutase [SOD], catalase [CAT], ascorbate peroxidase [APOX], and glutathione S-transferase [GST]), the phenoloxidase (PO) enzyme involved in melanogenesis and insect humoral immunity, and metallothionein (MT) levels of the fifth instar *Agrotis ipsilon*. Positive correlations are shown in red, while negative correlations are represented in blue, with the intensity indicating the strength of each relationship. This heatmap provides insights into the interactions between environmental and physiological stress factors in response to heavy metal exposure

strong positive correlations were observed among the antioxidant enzymes, with SOD showing particularly robust associations with APOX ($r=0.97$, $p<0.05$) and protein carbonyl ($r=0.96$, $p<0.05$). The oxidative stress markers protein carbonyl and lipid peroxides demonstrated a robust positive correlation ($r=0.98$, $p<0.05$), suggesting coordinated cellular responses to heavy metal-induced stress. Catalase exhibited significant negative correlations with most parameters, most notably with zinc ($r=-0.84$, $p<0.05$), lead ($r=-0.80$, $p<0.05$), and cadmium ($r=-0.79$, $p<0.05$). This inverse relationship suggests increased heavy metal concentrations may suppress CAT activity, potentially compromising the antioxidant defense system. Conversely, SOD and APOX showed strong positive correlations with most heavy metals, particularly copper (SOD-Cu: $r=0.81$; APOX-Cu: $r=0.87$, $p<0.05$), indicating their potential role in metal-induced oxidative stress response.

Metallothionein levels displayed moderate to strong positive correlations with GST ($r=0.90$, $p<0.05$) and Cr ($r=0.73$, $p<0.05$), suggesting its involvement in heavy metal detoxification processes. Phenoloxidase showed weak correlations with most parameters, with correlation coefficients ranging from -0.24 to 0.31 , indicating its relatively independent regulation in response to heavy metal stress. Among heavy metals, Zn, Pb, and Cd showed

strong intercorrelations ($r=0.85$ – 0.87 , $p<0.05$), suggesting similar accumulation patterns or shared uptake mechanisms. Nickel demonstrated relatively weaker correlations with other metals ($r=0.37$ – 0.67 , $p<0.05$), indicating potentially distinct accumulation or detoxification pathways. These correlation patterns provide insights into the integrated physiological responses of *A. ipsilon* larvae to heavy metal exposure, highlighting the complex interplay between antioxidant defense mechanisms, oxidative stress markers, and metal accumulation.

Light microscopy of ultrastructural changes in *A. ipsilon* midgut cells

In the midgut of larvae that consumed leaves from the reference site, the epithelial layer is surrounded by two distinct muscle layers: an outer longitudinal layer and an inner circular layer (Fig. 5a–c). The epithelial structure is composed of two main cell types: (1) columnar digestive cells, which feature a brush border of microvilli on their apical membrane facing both the peritrophic membrane and the gut lumen, and (2) regenerative cells, which can be found either individually or in small clusters “nidi” forming crypt-like structures.

Exposure to leaves from sites A and B induced notable ultrastructural alterations in the midgut cells of group 1 and group 2 larvae, indicating compromised cellular

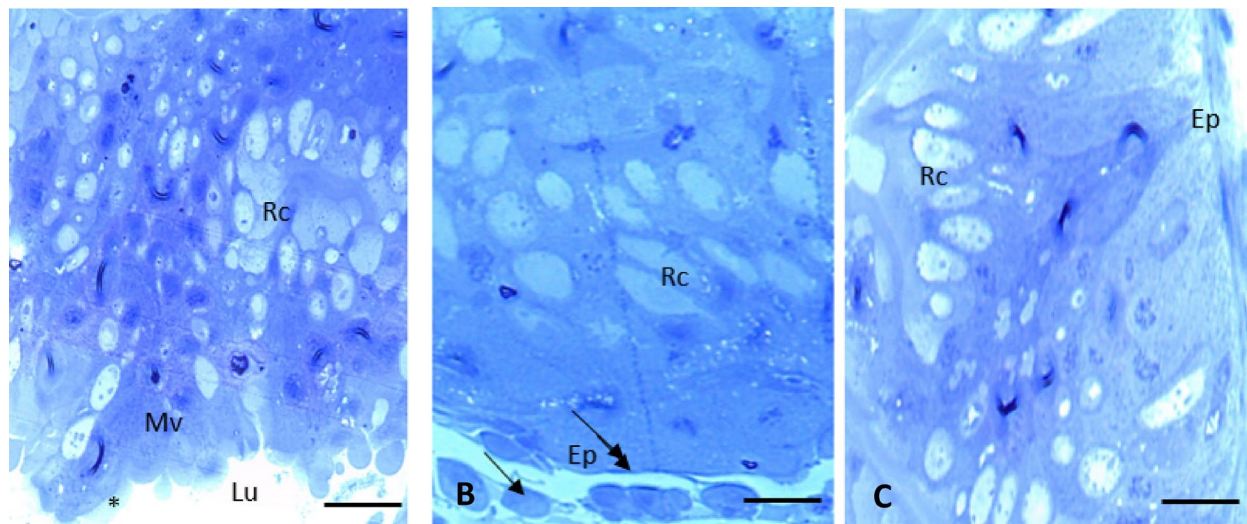


Fig. 5 a–c Semithin sections of midgut tissue from fifth-instar *Agrotis ipsilon* larvae of the reference group. The sections display a continuous epithelial sheath (EP) with well-organized microvilli (Mv) bordering the lumen (Lu) and a distinct peritrophic membrane (*). Regenerative cells (Rc) are located at the basal region of the epithelium, while the underlying longitudinal (arrow) and circular (double-headed arrow) muscle layers are clearly visible. Scale bar = 50 μ m

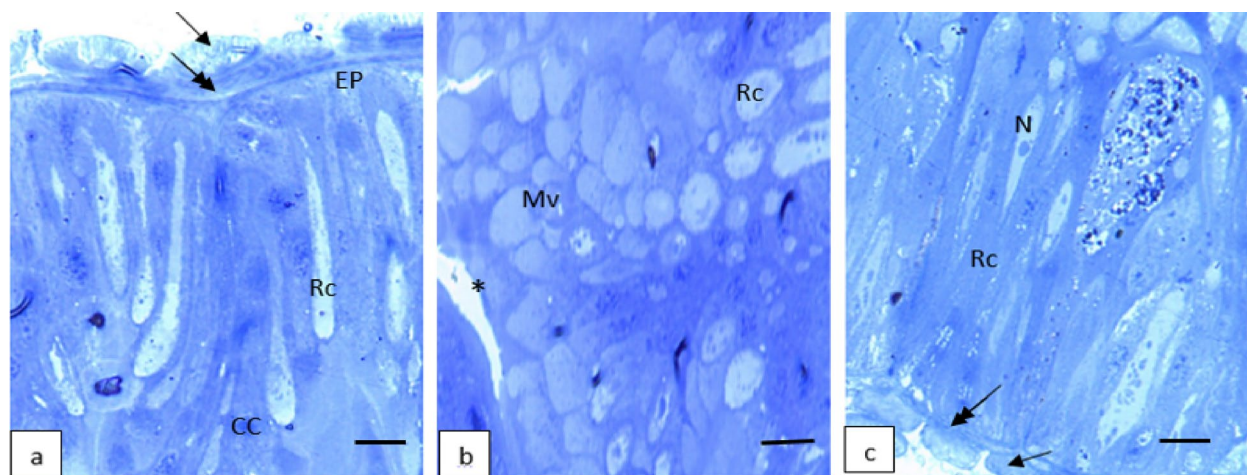


Fig. 6 a–c Semithin sections of midgut tissue from fifth-instar *Agrotis ipsilon* larvae fed on contaminated castor leaves. The epithelial sheath (EP) shows disorganization, and columnar digestive cells (CC) exhibit vacuolation and distorted microvilli (*). Regenerative cells (Rc) are irregularly distributed, and both longitudinal (arrow) and circular (double-headed arrow) muscle layers are visible beneath the epithelium. Scale bar = 50 μ m

integrity. Regenerative cells displayed deformed nuclei (Fig. 6a, c), and the brush border of microvilli in the columnar cells was visibly degraded (Fig. 6b). Additionally, intercellular spaces between adjacent cells were marked by incisions (Fig. 6b, c).

Midgut ultrastructural and observed changes in *A. ipsilon* larvae

The midgut, a primary organ for nutrient absorption, comprises a simple epithelial layer encased by longitudinal and circular muscle layers, as observed in larvae-fed leaves from the reference site. The columnar cells within the epithelium play a critical role in mineral bioaccumulation. These cells are anchored to the basal lamina,

with their apical ends extending into the gut lumen. The apical region features a dense arrangement of well-developed microvilli, followed by a basal zone containing parallel microtubules and small pinocytotic vesicles (Fig. 7A). Lysosomes containing mildly electron-dense materials were identified (Fig. 7A). The rough endoplasmic reticulum (rER) displayed parallel cisternal arrangements (Fig. 7B, D). In contrast, the basal regions of the cells were densely packed with mitochondria exhibiting clearly defined inner membranes (Fig. 7C).

Exposure to contaminated leaves caused notable damage in the midgut of group 1 and 2 larvae, with necrotic changes evident in the nuclei of regenerative cells, alongside apoptotic alterations in both regenerative and

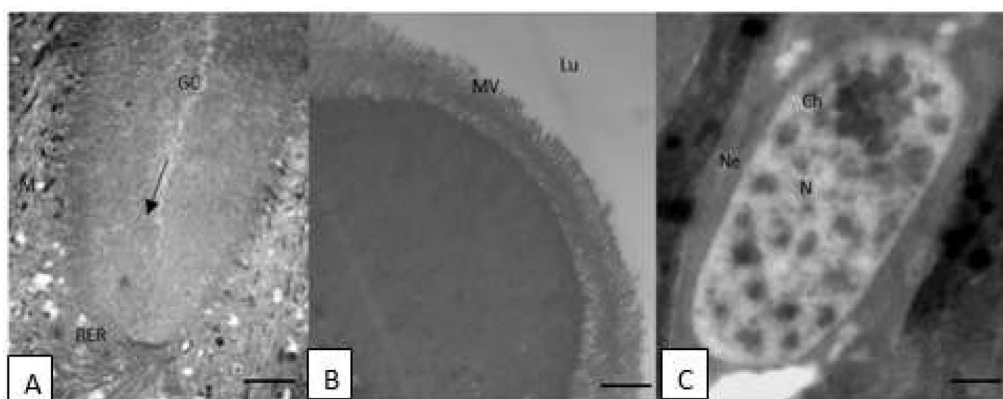


Fig. 7 Transmission electron micrographs of midgut tissue from the reference group of 5th instar *Agrotis ipsilon* larvae: **A:** A goblet cell displaying a goblet-shaped cavity, lateral cytoplasmic projections (arrow), mitochondria (M), and rough endoplasmic reticulum (RER) organized in parallel arrangements. Scale bar = 20 μ m. **B:** Microvilli (Mv) and lumen (Lu). **C:** Nucleus (N), nuclear envelope (Ne), chromatin (Ch), and mitochondria (M). Scale bar = 10 μ m

digestive cells (Fig. 8A, B). Goblet cells exhibited goblet-shaped cavities and fragmented lateral cytoplasmic projections (Fig. 8B, C, F). The presence of blebs was observed (Fig. 8E), along with irregular chromatin clumping within the nucleoplasm (Fig. 8A, D, E). Mitochondria in affected cells showed lysed matrices (Fig. 8F). Abundant lysosomes (Fig. 8A, B, F), electron-dense vesicles (Fig. 8A, E), and extensive cytoplasmic vacuolation were evident. Digestive cells also displayed increased spherite formation—membranous structures with concentric electron-dense layers—within the cytoplasm (Fig. 8G).

The findings provide compelling evidence of stress responses triggered by heavy metal exposure in *A. ipsilon* larvae feeding on contaminated *R. communis* leaves. Elevated protein carbonyl levels and intensified lipid peroxidation, particularly in midgut tissues, highlight substantial oxidative damage from direct contaminant exposure. Altered antioxidant enzyme activities, marked by high partial η^2 values (0.567–0.995), further underscore a robust biochemical response to metal-induced stress. A significant increase in metallothionein (MT) concentrations was observed in both midgut and brain tissues, with higher levels detected in the brain, suggesting active roles in metal sequestration and detoxification. These findings collectively support the potential application of *A. ipsilon* larvae as reliable bioindicators for monitoring heavy metal contamination in terrestrial ecosystems.

Discussion

Heavy metals induce ROS overproduction, disrupting the balance between ROS generation and scavenging and triggering oxidative stress through altered antioxidant enzyme activity (Coskun et al., 2021; Zhang et al., 2022). Environmental factors, including clay content, mineral composition, and organic matter, influence HM adsorption and bioavailability (Wuana & Okiyeimen, 2011),

while industrial emissions (metals, phosphates, sulfates, particulates) further amplify oxidative stress in exposed organisms (Shinkai et al., 2015; Yousef et al., 2017). We employed the polyphagous larva *A. ipsilon* as a sentinel of agroecosystem contamination, assessing dietary HM effects via oxidative damage markers (protein carbonyls, lipid peroxides) and changes in key antioxidant enzymes in midgut and brain tissues.

Larvae fed leaves from polluted sites showed elevated protein carbonyls and lipid peroxides relative to controls, in agreement with previous work (Lushchak, 2011; Yousef et al., 2017). Oxidative stress promotes protein carbonylation, which can break peptide chains and alter protein structure and function (Kelly & Mudway, 2003; Hermes-Lima, 2004), thereby impairing enzymatic activity and cellular regulation (Korsloot et al., 2004; Birben et al., 2012). The observed lipid peroxidation, a hallmark of oxidative stress, is consistent with studies using biomarkers such as isoprostanes and TBARS to quantify membrane damage (Lushchak, 2011; Birben et al., 2012).

Environmental metal pollutants directly and indirectly increase ROS production, triggering compensatory changes in antioxidant enzymes including APOX, CAT and SOD. SOD converts superoxide radicals to H_2O_2 , which CAT then decomposes to prevent H_2O_2 accumulation (Romero et al., 2000; Yousef et al., 2017). Cadmium in particular can affect antioxidant enzymes by altering SOD structure and reducing its activity, likely via depletion of Zn^{2+} required for SOD conformation (Huang et al., 2006). Downregulation of antioxidant enzyme expression under Cd exposure has also been reported (Chaitanya et al., 2014). Alterations of SOD, CAT, APOX and Cu-containing PPO under Cd stress were observed in *Oxya chinensis* (Lijun et al., 2005; Shukla et al., 2017), and CAT has been highlighted as a key enzyme in mitigating Cd-induced ROS in that species (Lijun et al., 2005). High Pb levels can denature enzyme activity after plant uptake

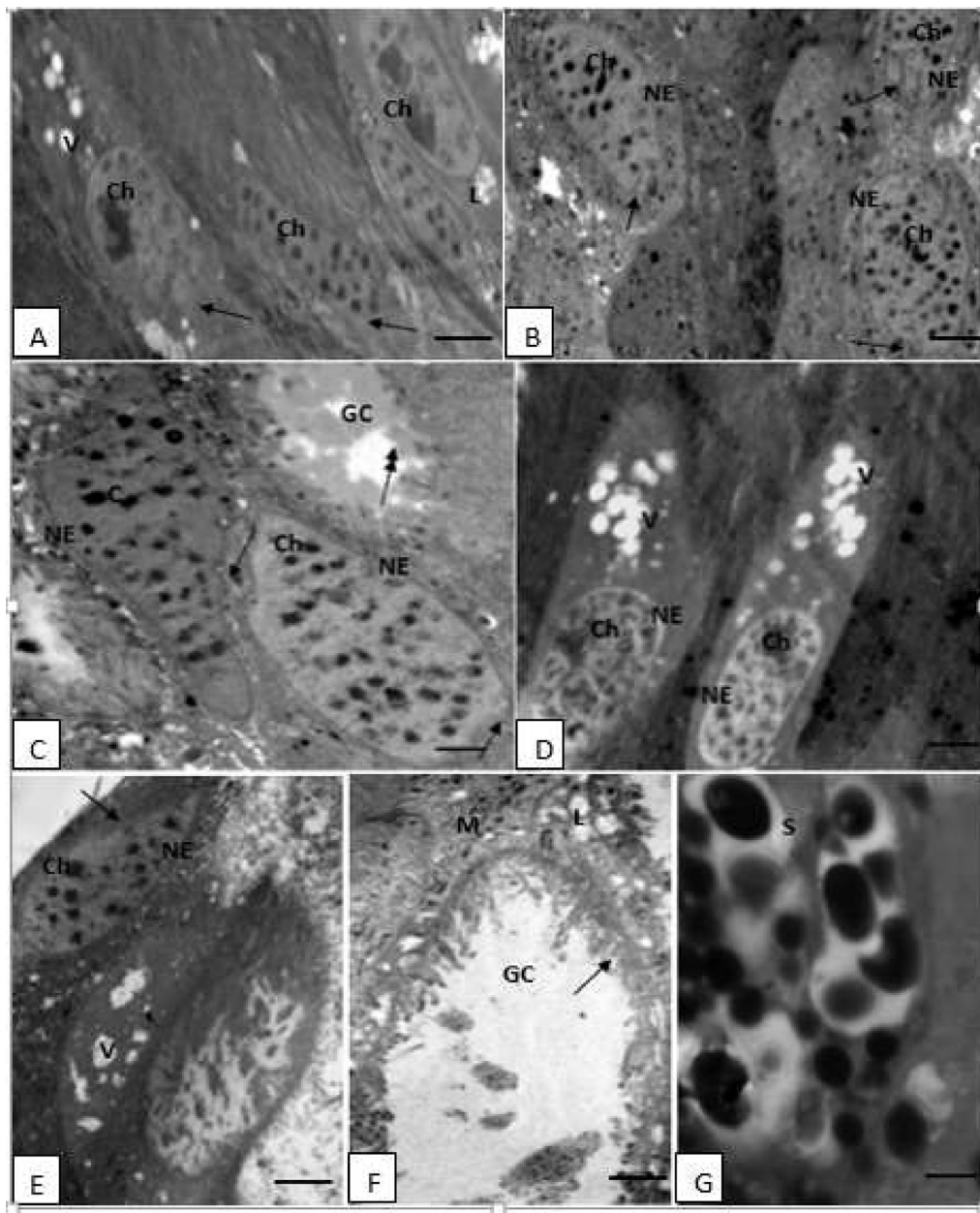


Fig. 8 Transmission electron micrographs of midgut cells from 5th instar *Agrotis ipsilon* larvae fed contaminated plant leaves from polluted sites **A** and **B**: **A**: Lysosomes (L), nuclear envelope necrosis (arrows), abnormal chromatin clumping (Ch), and vacuole (V). **B**: Intact nuclear envelope (arrows), nuclear envelope (NE), and chromatin clumps (Ch). **C**: A goblet cell (GC) with lysed cytoplasmic projections; the goblet-shaped cavity is intact, but lateral cytoplasmic projections are lysed (star), nuclear envelope remains intact (arrows), and chromatin clumps (Ch) are present. **D**: Abnormal chromatin clumps (Ch), nuclear envelope (NE), and vacuole (V). **E**: Abnormal chromatin clumps (Ch), nuclear envelope necrosis (arrows), and vacuole (V). **F**: A goblet cell showing a goblet-shaped cavity and lysed cytoplasmic projections (arrows), with mitochondria exhibiting lysed matrices (M) and lysosomes (L). **G**: Spherite (S). Scale bar = 10 μ m

via calcium channels and accumulation in shoots and leaves (López et al., 2007; Shu et al., 2015). APOX activity depends on ascorbate as the reducing agent for H_2O_2 , and environmental stress may limit ascorbate availability, impairing APOX function (Farooqui & Farooqui, 2011). HMs can also exert immunotoxic and immunomodulatory effects in insects and induce elevated PPO levels

(Pölkki et al., 2012; Nanda et al., 2019). Consistent with these reports, antioxidant enzyme activities (SOD, CAT, APOX and melanogenic PPO) were significantly higher in *A. ipsilon* larvae from polluted sites than in controls, which may reflect a trade-off between growth and antioxidant protection during development (Woodring & Sparks, 1987).

Long-term HM exposure likely selected for detoxification responses such as increased GST activity to counter oxidative damage (Zhang et al., 2014, 2022). In our study, GST activity was elevated in both midgut and brain tissues of larvae from polluted sites, consistent with findings in *O. chinensis* and *Poecilus cupreus* (Zhang et al., 2014, 2022). By contrast, Cd exposure reduced GST activity in female *D. melanogaster* (Hu et al., 2019), indicating that this response can be species- and sex-specific. The oxidative stress markers were generally more pronounced in gut than brain tissues, likely because the gut directly encounters ingested pro-oxidants, as reported for other phytophagous insects (Felton & Summers, 1995; Krishnan & Kodrik, 2006; Çelik et al., 2024). Heavy metal toxicity—particularly from Cd, Cu, Hg and Pb—often involves strong affinity for thiol-containing molecules, disrupting protein function and low-molecular-weight thiols (Oliveira et al., 2022).

Our results also underscore HM-induced cellular and histological damage in the midgut. The X-ray microanalysis confirmed elevated HM levels in plant material and in larval midgut tissues. The midgut, essential for nutrient absorption and ionic regulation, is especially vulnerable to HM damage (Ballan-Dufrancais, 2002). Structural and ultrastructural anomalies following HM exposure have been reported in insects and other invertebrates (Bednarska et al., 2009; Osman et al., 2015; Wang et al., 2019, 2020). In *A. ipsilon*, we observed chromatin clumping, nuclear envelope blebbing, and globular nucleoplasmic inclusions consistent with apoptosis, a process frequently linked to HM accumulation (Hay et al., 2004; Braeckman, 2011). Comparable apoptotic features—chromatin condensation and DNA fragmentation—were reported in *Heliothis virescens* midgut cells under Cd and Pb stress (Loeb et al., 2000).

The vacuolated cytoplasm we observed may reflect mitochondrial disruption or increased endocytic activity (Cavados et al., 2004; Polidori et al., 2018). Mitochondrial alterations, including matrix lysis and impaired membrane transport, further indicate HM cytotoxicity (Meyer et al., 2013). ER dilation, likely arising from HM interference with protein processing, can trigger ER stress responses (Li et al., 2024). Proliferation of myelin figures—often interpreted as an adaptive response to xenobiotic exposure—highlights the extent of cellular damage (Kheirallah, 2015). Microvillar disruption, the primary interface between the midgut and environmental toxins, corroborates earlier findings Gregory et al., 2002; Polidori et al., 2018). Deposition of electron-dense HM granules in the midgut epithelium is consistent with observations in mites (*Xenillus tegeocranus*) and paper wasps (*Polistes dominula*) from contaminated habitats (Pigino et al., 2006; Polidori et al., 2018).

We also detected increased mineral spherites in the midgut—structures implicated in HM detoxification and storage (Brown, 1982). These darkly stained spherites likely sequester toxic metals, and their proliferation correlated with reductions in lysosomal compartments, organelles central to cellular repair and detoxification (Lauverjat et al., 1989; Hopwood et al., 2023). Lysosomal responses to HM exposure, including changes in number and activity, represent a conserved cellular mechanism for managing sublethal injury (Zoncu & Perera, 2022). Collectively, these observations outline a pathway of HM-induced cellular injury, from structural alterations to organelle dysfunction.

This toxicopathology, driven by industrial emissions from fertilizer, cement and brick production, underscores the need for stricter environmental controls. Reducing industrial HM emissions could mitigate environmental contamination and its cascading impacts on ecological health (Wang et al., 2018; Bărbulescu & Hosen, 2025).

Conclusion

Dietary exposure to heavy metals in field-collected castor leaves caused pronounced biochemical and ultrastructural injury in fifth-instar *A. ipsilon* larvae. The midgut accumulated the highest metal burdens (notably Cd), displayed the greatest oxidative damage, and showed marked ultrastructural disruption (mitochondrial lysis, microvillar disorganization and chromatin condensation). Concurrent increases in antioxidant enzyme activities and metallothionein concentrations indicate activation of both enzymatic and thiol-mediated detoxification pathways. Together these biochemical and cellular endpoints provide robust, complementary evidence of metal-induced cytotoxicity and support the potential use of *A. ipsilon* as a practical terrestrial bioindicator for monitoring dietary metal contamination in agroecosystems. Future work should quantify temporal dynamics of accumulation and link sublethal cellular damage to fitness-relevant outcomes.

Abbreviations

ANOVA	Analysis of variance
APOX	Ascorbate peroxidase
CAT	Catalase
Cd	Cadmium
CDNB	1-Chloro-2,4-Dinitrobenzene
Cr	Chromium
Cu	Copper
DNPH	2,4-Dinitrophenylhydrazine
ER	Endoplasmic Reticulum
Gp1	Group 1 (Site A: Brick kiln emissions)
Gp2	Group 2 (Site B: Fertilizer/chemical effluents)
GSH	Reduced glutathione
GST	Glutathione S-transferase
HMs	Heavy metals
L:D	Light: dark cycle
L-DOPA	L-3,4-Dihydroxyphenylalanine
LP	Lipid Peroxides
MANOVA	Multivariate analysis of variance

MT	Metallothionein
Ni	Nickel
OD	Optical density
Pb	Lead
PC	Protein carbonyl
PBS	Phosphate-buffered saline
PO	Phenoloxidase
rER	Rough endoplasmic reticulum
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
TEM	Transmission electron microscopy
Tris-HCl	Tris hydrochloride
Zn	Zinc

Supplementary Information

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Supplementary Material 1

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Author contributions

The conception and design of the study were jointly developed by HAY, AM, and MS. SR, MS, and AM were responsible for material preparation, data collection, and analysis. The methodology was crafted by HAY, AM, and NK. The interpretation of the data was carried out by HAY, AM, MS, and NK. The project received supervision and guidance from HAY and AM. The initial manuscript draft was written by HAY, and all authors provided feedback on previous drafts. All authors reviewed and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable. This study did not involve any vertebrate animals or human participants. The experimental work was conducted on *Agrotis ipsilon*, a common agricultural insect pest, and does not require ethical approval. All authors have reviewed, comprehended, and adhered to the "Ethical Responsibilities of Authors" as outlined in the Instructions for Authors, where applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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